(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 January 2003 (23.01.2003)

PCT

(10) International Publication Number
WO 03/006653 A1

(51) International Patent Classification[7]: C12N 15/30, C12R 1/35, C07K 14/30, C12Q 1/68

(21) International Application Number: PCT/GB01/03128

(22) International Filing Date: 11 July 2001 (11.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0017073.8 11 July 2001 (11.07.2001) GB

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(81) Designated States *(national)*: JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR).

Published:
— *with international search report*

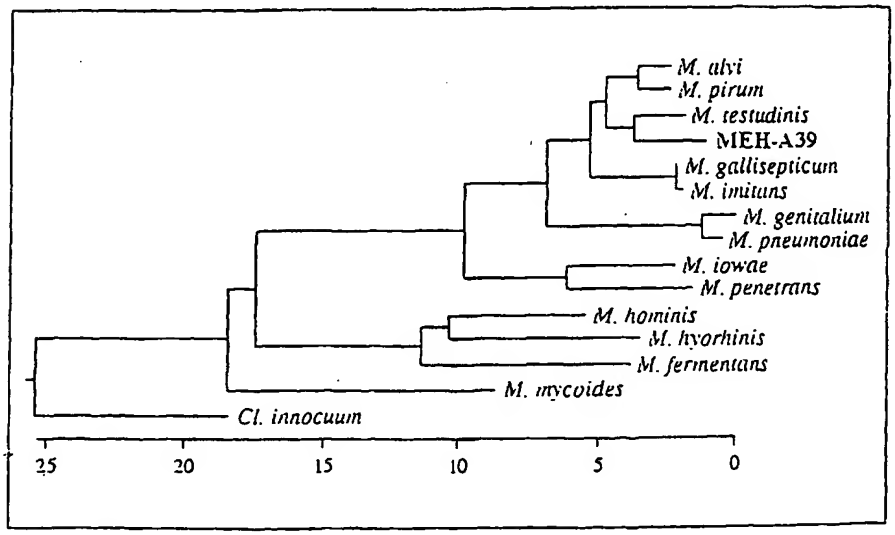
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

SEQ ID NO 2 in WO

(54) Title: MYCOPLASMA SPP. A39

Phylogenetic tree of 16S rDNA sequences

100% SEQ ID NO 4



(57) Abstract: An isolated mycoplasma spp A39, as deposited at the UK National Collection of Type Cultures under number 11740.

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MYCOPLASMA SPP. A39

The present invention relates to a microorganism of the mycoplasma type, assays therefor and treatment of mycoplasma infections.

Background to the Invention

Mycoplasmas are among the smallest and simplest known micro-organisms [1]. They measure between 0.2μm-0.3μm in size and have a genome size ranging from 600-1300kbp, and as such are the smallest organisms capable of independent existence. Phenotypically they are distinguished from other bacteria by the lack of cell wall component peptidoglycan and consequently they are not susceptible to (B-lactam anti-microbials such as penicillins and cephalosporins [2]. Taxonomically the genus Mycoplasma is member of the class Mollicutes (soft skin). There are more than a 100 Mycoplasma species currently recognised; they inhabit a wide range of ecological niches in animals, plants, insects, birds and reptiles, where they may act as commensals, pathogens or parasites [3].

Twelve species (M buccal, M fermentans, M faucium, M genitalium, M hominis, M lipophilium, M orale, M penetrans, M pneumoniae, M primatum, M salivarium and M spermatophilum) have been isolated from humans. M pneumoniae is the only species considered as an unequivocal primary human pathogen and it causes lower respiratory infections [4].

Mycoplasma hominis and M. genitalium have been isolated from patients with signs of respiratory and urogential

infection [5,6,7], and it is thought that they play a causal role in a proportion of these cases. There is considerable diagnostic confusion for other Mycoplasma species that make up part of the normal oropharangeal and urogenital flora. Thus the significance of the isolation of these species is uncertain unless achieved from a normally sterile site. The pathogenic mechanisms by which Mycoplasma can cause infections in certain individuals are not well understood [1,8]. Culture of Mycoplasma spp.is difficult as the organisms are slow growing, require specialist media and are readily overgrown and obscured by other organisms. Additionally poor understanding of the antigenic structure of these organisms confounds the serological diagnosis. These facts make it more difficult to understand the role of these agents in human infection either as primary pathogens or as opportunists.

Mycoplasma species are associated with several diseases and are often linked as cofactors in AIDS pathogenesis, to malignant transformation and auto-immune disease [8]. Mycoplasma fementans has been frequently isolated from normally sterile sites such as blood, urine and tissue of HIV infected individuals [9,10] and studies have shown that it is able to stimulate CD4+ lymphocytes and other immunomodulatory activities which could advance the onset of AIDS [1]. Other Mycoplasma species linked as cofactors to AIDS progression are M penetrans, a species detected almost exclusively in the urine of these individuals, M. genitalium and M pirum [8,12].

In recent years increasing evidence has emerged to support the role of Mycoplasma species in infections of immunocompromised patients, in particular to individuals with severe antibody deficiencies. The risk of infection in Primary Immunodeficiency, as well as in patients on prolonged immunosuppresive chemotherapy following organ transplantation, or for treatment for malignant diseases, is of growing concern to clinicians[8,12]. Antibody deficient individuals are especially prone to infections with Mycoplasma. Those with hypogammaglobulinemia can suffer slow progressive chronic infection of the respiratory tract and destructive arthritis [13]. Joint infections with the M pneumoniae, M salivarium and M hominis are also common in patients with hypogammaglobulinemia [14].

Mycoplasma species may have a role in the onset of autoimmune disease. Various species have been isolated from the joints of patients with rheumatoid arthritis, sexually transmitted reactive arthritis and other human arthritides [13,14]. A recent study using sensitive methods for detecting DNA identified M fermentans in the synovial fluid of 21% (8/38) patients with rheumatoid arthritis. It is possible mycoplasmal antigens can cross react with self antigens to trigger auto-immune rheumatic disease. Recently a super-antigen was identified in M. arthritis which induces an auto-immune response in mice [15].

Prior to 1980, bronchiectasis was the most common cause of death in Primary Antibody Deficient (PAD) patients [16]. There has since been a substantial reduction in morbidity

due to the introduction of intravenous immunoglobulin replacement therapy and the more effective use of antibiotics. However at least 50% of patients still suffer from progressive respiratory disease, and it has been suggested that members of the genus Mycoplasma may be an important cause [17].

Summary of the Invention

In a first aspect, the present invention provides an isolated mycoplasma spp A39. The applicants consider this mycoplasma to be a novel mycoplasma, which has been found to cause infection in a number of human subjects. A sample of mycoplasma spp A39 has been deposited at the UK National Collection of Type Cultures (NCTC) under accession number 11740 for the purposes of the present application. Samples of this mycoplasma are useful in developing assays for its detection, particularly so that appropriate therapy can be selected for its treatment.

In one embodiment, the mycoplasma spp A39 or a component thereof may be used as a control in an assay of a sample potentially containing the mycoplasma. Such assays include a morphological assay, an antibiotic sensitivity assay and a metabolism assay. The morphological assay may comprise comparing the morphology of a microorganism suspected to be mycoplasma spp A39 with that of authentic mycoplasma spp A39 on a solid growth medium where colonies are compared, or in a culture medium such as a liquid culture suitable for microscopic comparison of the mycoplasma itself. A suitable antibiotic sensitivity assay may compare the microorganism suspected to be mycoplasma spp A39 with

authentic mycoplasma spp A39 by their ability to grow on various media containing appropriate concentrations of antibiotics. A suitable metabolism assay compares the ability of each microorganism to metabolise various nutrients such as glucose, arginine and triphenol tetrazolium chloride (TTC), or urea. Enzyme activities such as phosphotase activity may be compared. A further assay comprises a nucleotide sequence assay where the length and/or sequence of a characteristic sub-sequence from the genome of the microorganism is compared with that of mycoplasma spp A39. Each of these assays may be used on their own or in combination with one another.

As to sample selection, any suitable body fluid may be used as a sample, depending on the nature and site of suspected infection. In particular, where infection is suspected in the respiratory tract a sputum sample is appropriate.

In a further aspect, the present invention provides a method for assaying for mycoplasma spp A39, which comprises:

(i) culturing a sample potentially containing mycoplasma spp A39 on a solid phase culture medium capable of supporting growth of mycoplasma spp A39 so as to produce individual colonies; and

(ii) identifying colonies as mycoplasma spp A39 where (a) no central spot in the colony is detected, (b) where the diameter of the colonies is less than about 0.3mm, preferably in the range 0.1 to 0.3mm, and/or (c) where the colony appearance is

granular. The growth time for assaying colony morphology is generally about five days.

Additionally, or alternatively, a method of assaying for mycoplasma spp A39 is provided which comprises preparing an isolated sample of mycoplasma spp A39 for electron microscopy and identifying the mycoplasma as mycoplasma spp A39 where individual mycoplasma are flask shaped.

In a further aspect, the present invention provides a method for treating a mycoplasma infection in a subject in need of such treatment. The method comprises administering to the subject a therapeutically effective amount of an antibotic capable of treating infection by mycoplasma spp A39.

Accordingly, the invention provides use of a compound capable of treating infection by mycoplasma spp A39, for the production of an antibiotic composition for the treatment of a subject with a mycoplasma spp A39 infection. The subject may be a human, more particularly an immunocompromised human, such as a patient with AIDS or hypogammaglobulinemia.

Typically, the subject is capable of producing a sample, such as a sputum sample, which is positive in an assay for mycoplasma spp A39 or a component thereof. Such assays include those detailed herein. In accordance with the present invention it is advantageous to perform an assay on a sample subject suspected of having a mycoplasma infection to test whether the mycoplasma is spp A39. If the assay is

positive, some conventional antibiotic treatments would not be expected to be effective and rather than waste resources on an inappropriate antibiotic treatment, a more appropriate antibiotic capable of treating infection by mycoplasma spp A39 can be selected.

Accordingly, in a further aspect, the invention provides a method of selecting an agent for treating a mycoplasma infection in a subject, which method comprises:

(i) obtaining a sample from the subject and assaying the sample for mycoplasma spp A39; and

(ii) where the assay is positive, selecting as an agent for treating the infection a pleuromutilin comprising valnemulin or an analogue or derivative thereof.

In a further aspect, the invention provides an oligonucleotide probe which is capable of hybridising to a region of polynucleotide from mycoplasma spp A39 and substantially incapable of hybridising to a polynucleotide or the corresponding region thereof from a further mycoplasma, typically under substantially the same stringency of hybridisation conditions. In this way, the oligonucleotide probe provides a basis for an assay for mycoplasma spp A39 which is capable of discriminating between this mycoplasma and at least one further mycoplasma species. Preferably, the further mycoplasma is selected from one or more of the following mycoplasma species: imitans, hominis, fermentans, genitalium, pneumoniae, pirum, testudinis, alvi, gallisepticum, vulis and penetrans. More preferably, the oligonculeotide probe is

incapable of hybridising to any of those further mycoplasmas and, most preferably, to all other mycoplasma species. The exact length and sequence of the oligonucleotide probe will depend upon desired specificity of the probe (in discriminating between mycoplasma spp A39 and other species). The polynucleotide may be DNA or RNA and may comprise an amplified region from the mycoplasmic genome. In one embodiment, the oligonucleotide probe is selected so as to be capable of hybridising to the 16s rDNA region. The 16s rDNA region is generally as defined in either Table 2 or Table 3 below. Suitable probes may be designed by comparing the alignment of the various sequences from different mycoplasma. Where, for example, a probe unique to mycoplasma spp A39 is required, a sub-sequence of the region of polynucleotide is selected which is unique to A39 and different in the other mycoplasma species.

A typical probe length is at least 15 nucleotides and preferably not more than 30 nucleotides. A preferred length is in the range from 15 to 20 nucleotides. Such oligonucleotide probes may be prepared by any conventional method and would typically further comprise a label to facilitate detection, such as a fluorescent or radiolabel.

In a further aspect, the present invention provides a kit for assaying mycoplasma spp A39, which kit comprises an oligonucleotide probe as defined herein, together with a suitable buffer system.

In a further aspect, the present invention provides an oligonucleotide primer pair for use in a polymerase chain reaction (PCR), which pair is capable of amplifying in PCR a target nucleic acid sequence from mycoplasma spp A39 and incapable of amplifying a sequence of the same length on the target sequence from a further mycoplasma. In this way, the oligonucleotide primer pair provides a basis for an assay for mycoplasma spp A39 which is capable of discriminating between this mycoplasma and at least one further mycoplasma species. The PCR-based assay may be specific to mycoplasma spp A39 either on the basis of the size of target sequence amplified, which can be selected to be different in the further mycoplasma, or on the basis that no target sequence is amplified at all in the further mycoplasma. In the latter case, it is preferred that at least one of the pair is capable of hybridising to a region of polynucleotide from the mycoplasma spp A39 and substantially incapable of hybridising a polynucleotide, or a corresponding region thereof, from the further mycoplasma. In the absence of hybridisation by one or both of the pair of oligonucleotide primers, no amplification would be expected.

The further mycoplasma is preferably defined as set out above in relation to the oligonucleotide probes. As with the probes, the exact length and sequence of the oligonucleotide primer pair will depend upon the desired specificity of each member. In one embodiment, at least one member of the pair is selected so as to be capable of hybridising to the 16s rDNA region. Suitable primers may therefore be designed by comparing the alignment of the

various sequences from different mycoplasma in the same way as a suitable oligonucleotide probe may be designed. A typical primer length is at least 15 nucleotides and preferably not more than 35 nucleotides. A preferred length is in the range from 20 to 30 nucleotides. A particularly preferred primer pair is:

amphf	5'	AAG CTA GTA AAG GAA ATG TTA TT	3'
amphr	5'	TCG ACT ATA TTT CTA TAG TTT TG	3'

In a further aspect, the present invention provides a kit for a PCR assay for mycoplasma spp A39. The kit comprises an oligonucleotide primer pair as defined herein, and one or more further components selected from a DNA polymerase and a suitable buffer system.

In a further aspect, the present invention provides a method for assaying for mycoplasma spp A39, which comprises:

(i) obtaining a sample potentially containing mycoplasma spp A39;

(ii) determining the length of the 16S-23S intergenic spacer sequence; and

iii) identifying mycoplasma spp A39 where the length of the 16S-23S intergenic spacer sequence is around 430 bp. The length of 16S-23S intergenic spacer sequence is preferably determined by amplifying that region of the DNA typically by PCR and determining the length by any conventional means, such as using agarose gel electrophoresis.

Brief Description of the Drawings

The invention will now be described in further detail, by way of example only, with reference to the accompanying drawings, in which:

FIGURE 1 shows colonies of mycoplasma according to the present invention in comparison to other known mycoplasma;
FIGURE 2 shows morphology of the mycoplasma of the present invention;
FIGURE 3 shows a phylogenetic tree of 16S rDNA sequences of mycoplasma; and
FIGURE 4 shows differences in the 16S-23S intergenic spacer sequence of various mycoplasma, using agarose gel electrophoresis.

Detailed Description of the Invention

Example 1

Isolation of novel mycoplasma and antibiotic treatment

Purulent sputum from a 30 year old patient (DC) with X-linked agammaglobulinaemia, bronchiectasis and chronic bronchitis gave consistently negative results on routine culture. An unusual Mycoplasma (designated A39-see below) was isolated in high concentration on four separate occasions over a three month period from this patient's sputum, despite treatment with doxycycline and ciprofloxacin. The minimal inhibitory concentrations (MICs) for the organism were 0.2ug/ml for doxycycline, 1.5ug/ml for ciprofloxacin and <0.1ug/ml for a novel pleuromutilin antibiotic Econor - Registered Trademark of Novartis, which now has a licence for treating mycoplasma pneumonia in pigs

[19]. This antibiotic is known generically as valnemulin. Compassionate five day treatment with Econor lead to rapid resolution of the patient's symptoms, reduction in a high neutrophil count and normalisation of serum C reactive protein, and a negative sputum culture. The bronchitis relapsed two months later, the sputum again being positive, and a further 10 day course of Econor was followed by rapid improvement and a negative culture. Subsequently, sputum cultures from three further PID patients with Common Variable Immunodeficiency (from about 25 screened) with chronic bronchitis were positive for the same Mycoplasma (designated A39).

All four isolates from the four separate patients produced distinct and unusual colonies (see Figures 1 and 2) when grown on special solid media[20] (obtainable from Mycoplasma Experience Ltd of Reigate, Surrey UK as "solid mycoplasma medium"), and did not have the classical 'fried egg' appearance of M pneumoniae. Biochemical tests confirmed (table 1). Electron microscopy of the original isolate showed flask shaped organisms with trilaminar membranes and a denser area at the apex suggesting an adhesion site (Fig 2). The organism is morphologically similar to M pneumoniae and M pirum.

Table 1

	A39	*pneumoniae*	genitalium	hominis	fermentans
Cell Morphology (EM)	Flask shaped with trilaminar membrane	Mulberry shaped	Spherical or pleomorphic with translaminar membrane		
Biochemical Reactions					
glucose	+	+	+	-	+
arginine	-	-	-	+	+
TTC (aerobic)	+	+	+	-	-
phosphotase	-	-	-	-	+/-
urea	-	-	-	-	-
Time to Recovery (days)	3-10	4-21	slow	1-5	5-10

Example 2

Nucleotide Sequence Assays

Assays based on differences in the DNA sequence between various mycoplasma can be developed so as to identify the presence of mycoplasma spp A39 in a sample.

DNA amplification methods using the polymerase chain reaction (PCR) have facilitated the rapid detection of low numbers of pathogens from clinical specimens, and may be applied with success to the detection of mycoplasmas from clinical samples. Amplification of the 16Sr RNA gene may be routinely performed. The 16S rRNA gene is found in all prokaryotes and consists of regions of sequence which are highly conserved within a particular genus, interspersed with regions of sequence which vary between species. The variable regions may be utilised to design either species

specific amplification primers or DNA probes. The ribosomal 16S region of A39 was cloned and sequenced. 16S rDNA analysis was carried out as described in Ref 21. Sequence data processing used UPGMA clustering, PHYLIP, bootstrapping and consensus algorithms. PCRs for *M.pneumoniae*, *M. genitalium* and *M. fermentans* were carried out.

Alignment and cluster analysis of the 16S rDNA places A39 in the *M. pneumoniae* group with highest homology to *M. testudinis* (93%) and *M. pirum* (92%)(Fig. 3). PCRs for *M. pneumoniae*, *M. genitalium* and *M. fermentans* were negative.

Amplification of the internal transcribed spacer region was also performed. The 16S-23S spacer length for A39 was 430bp, which is quite distinct in size from other mycoplasma species (Fig. 4).

The present PCR assay targets a Mycoplasma genus specific region of the 16s rRNA gene together and an internal positive control to monitor each reaction. Analysis of the labelled product is performed by solution hybridisation to capture probes in a microtitre plate format, which in turn are labeled with biotin to allow hybridisation to streptavidin-coated plates. Multiple sequence alignments for A39, when compared to other Mycoplasma 16S rRNA genes, suggests that there are regions that could be targeted to design a species-specific molecular diagnostic assay. Using this sequence information a range of specific DNA probes for A39 and other important human mycoplasmas may be designed for diagnostic use. In these tests, the amplification of labeled genus specific product could be

hybridised in separate reactions with species specific DNA probes. Non-specific products not bound to the capture probe/microtitre plate would be removed during the washing stages. Only the specific bound hybrid would be detected using a colorimetric detection system.

The results of sequence alignment studies are presented in Table 2 below.

Table 2

Pairwise Sequence Alignment Comparing the 16S Gene of A39 with Mycoplasma alvi

>gb|U44765.1|MSU44765 Mycoplasma alvi 16S ribosomal RNA (rrn) gene, partial sequence
Length = 1458

Score = 2050 bits (1034), Expect = 0.0
Identities = 1363/1457 (93%), Gaps = 13/1457 (0%)
Strand = Plus / Plus

```
Query: 47   ctggcggcatgcctaataccaatgcaagtcgatcgggtgcagcaatgcatcagaggcgaa 106
            ||||||||||||||||||  ||||||||||||||| | |||||| |   |||||||||
Sbjct: 1    ctggcggcatgcctaatac--atgcaagtcgatcgggcgtagcaatacgctagaggcgaa 58

Query: 107  cgggtgagtaacacgtatccaatttacccccatagcgagggataacttagtcgaaagatt 166
            |||||||||||||||||||||||| |||||| | || | |||| |||| ||||||||||||
Sbjct: 59   cgggtgagtaacacgtatccaatctaccccaa-agtgggggacaact-agtcgaaagatt 116

Query: 167  agctaataccgcataacgattaaaatatcgcatgagaattaatttaaagatccgtttgga 226
            ||||||||||||||||  | |  | ||||||||||||||  | |||||||  ||||||||||
Sbjct: 117  agctaataccgcataataaatgcactatcgcatgagaagcattttaaaggtccgtttgga 176

Query: 227  ttactatgggataagggtgcggcatatcagctagttggtgaggtaacggcccaccaaggc 286
               |||||||||  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 177  ccgctatgggatgagggtgcggcatatcagctagttggtgaggtaacggcccaccaaggc 236

Query: 287  aatgacgtgtagttatgctgagaggtagaataaccacaatggaactgagacacggtccat 346
             |||||||||||||||||||||||||||||||||||||||||||||| |||||||||||| ||||
Sbjct: 237  gatgacgtgtagttatgctgagaggtagaataaccacaatgggactgagacacggcccat 296

Query: 347  actcctacgggaggcagcagtagggaatttttcacaatggacgaaagtctgatggagcaa 406
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Sbjct: 297  actcctacgggaggcagcagtagggaatttttcacaatggacgaaagtctgatggagcaa 356

Query: 407  tgccgcgtgaacgatgaaggtctttgtagattgtaaagttcttttatttaggaagaaaag 466
Sbjct: 357  tgccgcgtgaacgatgaaggtcttaatggattgtaaagttcttttatttgggaagaattg 416

Query: 467  ctagtaaaggaaatgttattagtttgaccgtactatttgaataagtaacgactaactatg 526
Sbjct: 417  ccaatagaggaaatgctattggtttgacggtaccatttgaataagtaacgactaactatg 476

Query: 527  tgccagcagtcgcggtaatacataggttgcaagcgttatccggatttaattgggcgtaaa 586
Sbjct: 477  tgccagcagtcgcggtaatacataggttgcaagcgttatccggattta-ttgggcgtaaa 535

Query: 587  acaagcgcaggcggattagaaagtctggtgttaaaatcaactgcttaacggt-gaatgca 645
Sbjct: 536  gcaagcgcaggcggattagaaagtctggtgttaaaaacaactgcttaacggttgtatgca 595

Query: 646  ttggaaacttctagtctagagtgtggtagagagttctggaactccatgtggagcggtgga 705
Sbjct: 596  ttggaaacttctagtctagagtgtggtagagagttctggaactccatgtggagcggtgga 655

Query: 706  atgcgtagatatatggaagaacaccagctgcgaaggcgagaacttaggccattactgacg 765
Sbjct: 656  atgcgtagatatatggaagaacaccagttgcgaaggcgagaacttaggccattactgacg 715

Query: 766  cttaggcttgaaaagtgtggggagcaaataggattagataccctagtagtccacaccgta 825
Sbjct: 716  cttaggcttgaa-agtgtggggagcaaataggattagataccctagtagtccacaccgta 774

Query: 826  aacgatggatgttaaacgtcggggcgatcacctcggtgttgcagttaacacattaaacat 885
Sbjct: 775  aacgatggatgttaggtgtcggggcgatcacctcggtgccgcagctaacgcattaaacat 834

Query: 886  cctgcctgggtagtacattcgcaagaatgaaactcaaacggaattgacggggacccgcac 945
Sbjct: 835  cccgcctgggtagtacattcgcaagaatgaaactcaaacggaattgacggggacccgcac 894

Query: 946  aagtggtggagcatgttgcttaattcgacggtacacgaaaaaccttacctagacttgaca 1005
Sbjct: 895  aagtggtggagcatgttgcttaattcgacggtacacgaaaaaccttacctagatttgaca 954

Query: 1006 tccttggcaaaactatagaaatatagtcgaggttaaccgaaagacaggtggtgcatggtt 1065
Sbjct: 955  tccttggcaaagctatagaaatatagtggaggttaaccgagtgacaggtggtgcatggtt 1014

Query: 1066 gtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttatcg 1125
Sbjct: 1015 gtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttttcg 1074
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Query: 1126 ttagttactttgtctaacgagactgccaacgcaagttggaggaaggtggggatgacgtca 1185
           |||||||||||||||| ||| ||||||||||||||||||||||||||||||| |||||||
Sbjct: 1075 ttagttactttgtctagcgatactgccaacgcaagttggaggaaggtggggacgacgtca 1134

Query: 1186 aatcatcatgccccttatgtctagggctgcaaacgtgctacaatggccaatacaaacagt 1245
           |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct: 1135 aatcatcatgccccttatgtctagggctgcaaacgtgctacaatggccattacaaacagt 1194

Query: 1246 taccaaaccgtaaggtggagttaatctgcaaagttggtctcagttcggattgagggctgc 1305
           | | || ||| ||||||||| |||||||||||| |||||||||||||||||||||||||
Sbjct: 1195 tgcaaatccgcaaggtggagctaatctgcaaagatggtctcagttcggattgagggctgc 1254

Query: 1306 aattcgccctcatgaagtcaggaatcactagtaatcgcgaatccagctatgtcggcggtga 1365
           |||||||||||||||||| |||||||||||||||||||| |||| ||||||||||||
Sbjct: 1255 aattcgccctcatgaagtc-ggaatcactagtaatcgcgaat-cagccatgtcgcggtga 1312

Query: 1366 atacgttctcgggtcttgtacacaccgcccgtcaaactacgagagttgatagttgtctaa 1425
           |||||||||||||||||||||||||||||||||||||||| ||||| || ||  | |||||
Sbjct: 1313 atacgttctcgggtcttgtacacaccgcccgtcaaactatgagagctggta-atatctaa 1371

Query: 1426 aaccgtgttgctaacccgcaaggaagcgcatgtctagggacaagattaatgattgggagt 1485
           |||||||||||||| ||| |||||||||||||||||||  | || |  ||||| |||||
Sbjct: 1372 aaccgtgttgctaa-ccgtaaggaagcgcatgtctaggg-taggactggtgatt-ggagt 1428

Query: 1486 taagtcgtaacaaggta 1502
           |||||||||||||||||
Sbjct: 1429 taagtcgtaacaaggta 1445
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Ribosomal DNA is present in multiple copies within the genome and therefore is a highly attractive target for sensitive diagnostic PCR assays. The 16S gene consists of regions of nucleotide sequences which are highly conserved interspersed with blocks of sequence which show variability. The gene has evolved slowly and is useful for evolutionary studies. The internal transcribed spacer (ITS) regions however evolve the fastest and therefore show the greatest differences between the species. Interspecific variation has been demonstrated within the ITS regions for other micro-organisms including Mycoplasma species (23). Sequence variation within the 16S rDNA has also been used

successfully for discriminating between species and forms the basis of diagnostic PCR assays (24,25).

The new isolate has been shown to be significantly different from the other species of Mycoplasma using Genetic Computer Group (GCG)package to access and compare other Myoplasma sequences from GenBank. Amplification of the ITS has also been perfomed for A39. The amplification product was found to be distinct in size from the other human Mycoplasma species also tested.

Various computer software packages are available to assist in the design of PCR primers.

Sequence analysis programs for Primer design include; Primer v1.01; Oligo, Cambridge Biosciences; Prime (GCG(National Library of Medicine, Washington, D.C.).

Sequence alignments and homology searches may be performed using programme BLAST version 2.0; PileUp; Clustalv (GCG(National Library of Medicine, Washington, D.C.

Example 3

Further Clinical Studies

Table 3 summarises data from tests on patients who have primary antibody deficiencies, acute chest infections (pneumonia) or chronic bronchitis. Sputum samples from each patient were cultured where indicated and subjected to PCR using the amphf/amphr primers described above. A comparison was made with throat swabs from normal subjects.

The absence of detectable A39 in normal subjects suggests that A39 is not a commensal and may be a pathogen.

Table 3

Type of patient	No tested	PCR pos for A39	Culture positive
Primary antibody deficiency	40	8	5 out of 6 tested
Community pneumonia	50	-	not done
Chronic bronchitis	20	1	not done
Normal subjects (throat swab)	52	0	not done

Discussion

The "flask-shaped" morphology and hydrolysis of glucose suggest that this organism could be related to *M. pneumoniae* and this is confirmed by 16S rDNA sequencing. However, homology with *M. pneumoniae* was 86% and MEH-A39 appeared and to be more closely related to *M. pirum* and *M.testudinis*. The length of the 16S-23S spacer sequence (430bp) was much longer than for other species of mycoplasma in the clade with the exception of *M. gallisepticum* (*ca* 650bp), a species reported to have an atypical RNA operon gene arrangement (Harasawa et al. 1992).

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SEQUENCE LISTING

<110> University College London

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CLAIMS:

1. An isolated mycoplasma spp A39, as deposited at the UK National Collection of Type Cultures under number 11740.

2. Use of mycoplasma spp A39 or a component thereof as a control in an assay of a sample potentially containing mycoplasma spp A39.

3. Use according to claim 2, wherein the assay comprises one or more of the following: a morphological assay, an antibiotic sensitivity assay, and a metabolism assay.

4. Use according to claim 2, wherein the assay comprises a nucleotide sequence assay.

5. Use according to claim 4, wherein the nucleotide sequence assay comprises a hybridisation probe assay or a PCR-based assay.

6. Use of a compound capable of treating infection by mycoplasma spp A39, for the production of an antibiotic composition for the treatment of a subject with a mycoplasma spp A39 infection.

7. Use according to claim 6, wherein the subject is a human.

8. Use according to claim 6 or claim 7, wherein the subject is capable of producing a sample which is positive in an assay for mycoplasma spp A39 or a component thereof.

9. Use according to claim 8, wherein the assay comprises one or more of the following: a morphological assay, an antibiotic sensitivity assay, and a metabolism assay, or a nucleotide sequence assay.

10. Use according to any one of claims 6 to 9, wherein the compound comprises a pleuromutilin.

11. Use according to claim 10, wherein the pleuromutalin comprises valnemulin or an analogue or derivative thereof.

12. A method of selecting an agent for treating a mycoplasma infection in a subject, which method comprises:

(i) obtaining a sample from the subject and assaying the sample for mycoplasma spp A39; and

(ii) where the assay is positive, selecting as an agent for treating the infection a pleuromutalin comprising valnemulin or an analogue or derivative thereof.

13. An oligonucleotide probe which is capable of hybridising to a region of polynucleotide from mycoplasma spp A39 and substantially incapable of hybridising to a polynucleotide or corresponding region thereof from a further mycoplasma.

14. An oligonucleotide probe according to claim 13, wherein the further mycoplasma is selected from one or more of imitans, homonis, fermentans, genitalium, pneumoniae, pirum, testudinis, alvi, gallisepticum, vulis and penetrans.

15. An oligonucleotide probe according to claim 14, wherein the further mycoplasma comprises all other mycoplasma species.

16. An oligonucleotide probe according to any one of claims 13 to 15, wherein the region of polynucleotide comprises the 16s rDNA region

17. An oligonucleotide probe according to claim 16, wherein the 16s rDNA region is as defined in Table 2 or Table 3.

18. A kit for assaying mycoplasma spp A39, which comprises an oligonucleotide probe according to any one of claims 13 to 17, and a buffer system.

19. An oligonucleotide primer pair for use in a polymerase chain reaction (PCR), which pair is capable of amplifying in PCR a target nucleic acid sequence from mycoplasma spp A39 and incapable of amplifying a sequence of the same length on the target sequence from a further mycoplasma.

20. An oligonucleotide primer pair according to claim 19, wherein at least one of the pair is capable of hybridising to a region of polynucleotide from mycoplasma spp A39 and substantially incapable of hybridising to a polynucleotide or corresponding region thereof from the further mycoplasma.

21. An oligonucleotide primer pair according to claim 20, wherein the region of polynucleotide comprises the 16s rDNA region.

22. An oligonucleotide primer pair according to claim 21, wherein the 16s rDNA region is as defined in Table 2 or Table 3.

23. An oligonucleotide primer pair according to any one of claims 19 to 22, wherein the further mycoplasma is selected from one or more of imitans, homonis, fermentans, genitalium, pneumoniae, pirum, testudinis, alvi, gallisepticum, vulis and penetrans.

24. An oligonucleotide primer pair according to any one of claims 19 to 23, wherein the further mycoplasma comprises all other mycoplasma species.

25. A kit for a PCR assay for mycoplasma spp A39, which comprises an oligonucleotide primer pair according to any one of claims 19 to 24, and one or more further components selected from a DNA polymerase and a buffer system.

26. A method for assaying for mycoplasma spp A39, which comprises:

(i) obtaining a sample potentially containing mycoplasma spp A39;

(ii) determining the length of the 16S-23S intergenic spacer sequence; and

iii) identifying mycoplasma spp A39 where the length of the 16S-23S intergenic spacer sequence is around 430 bp.

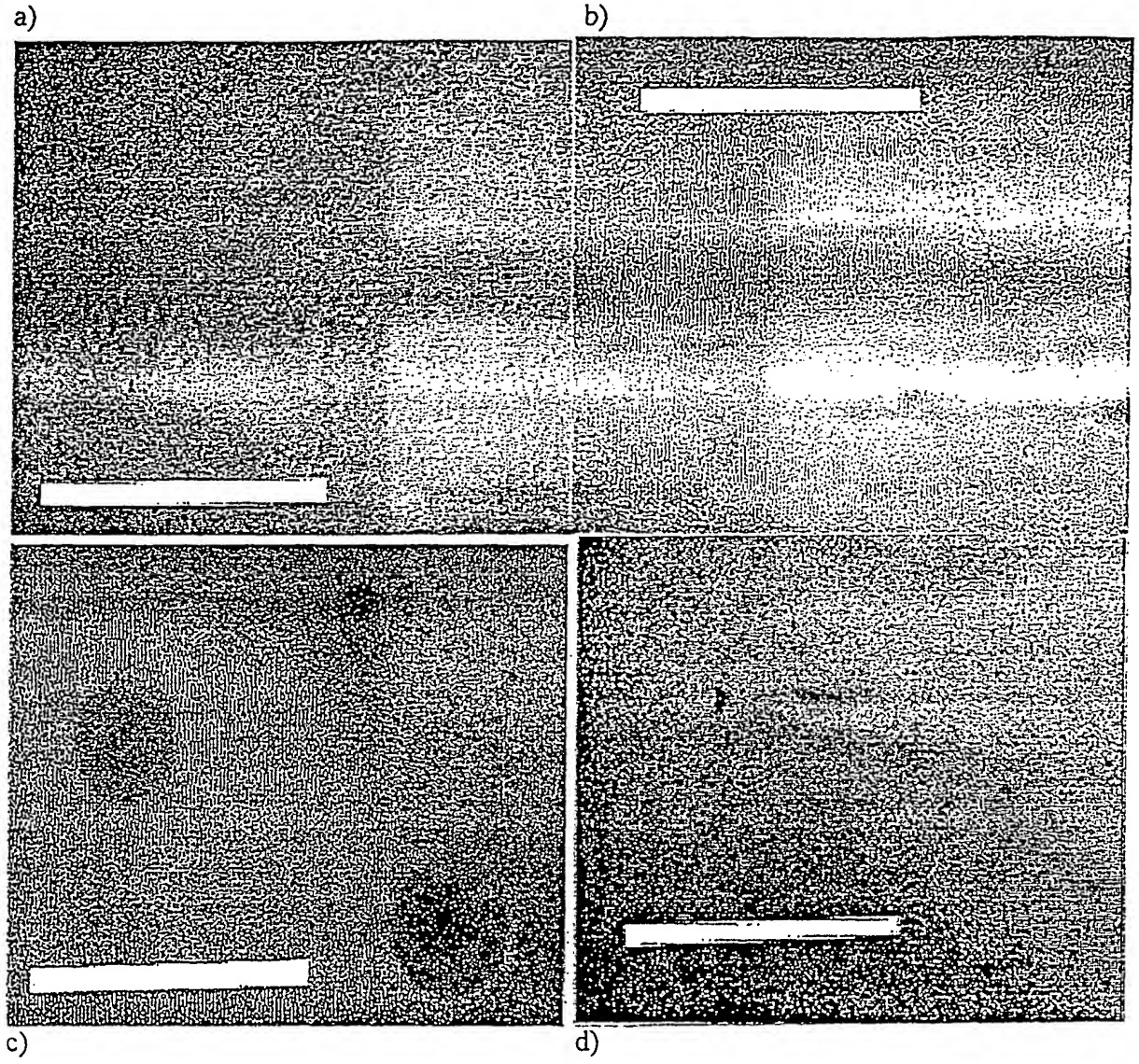
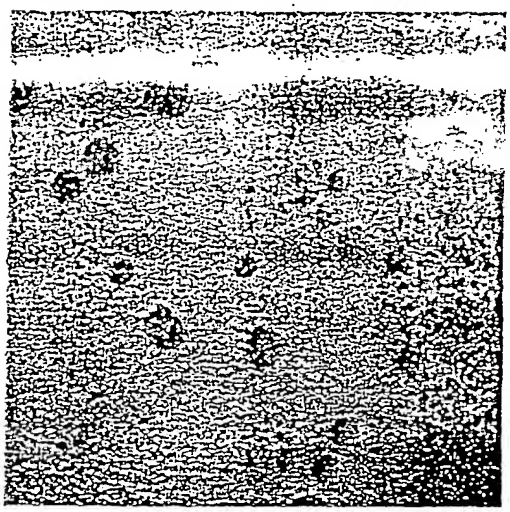


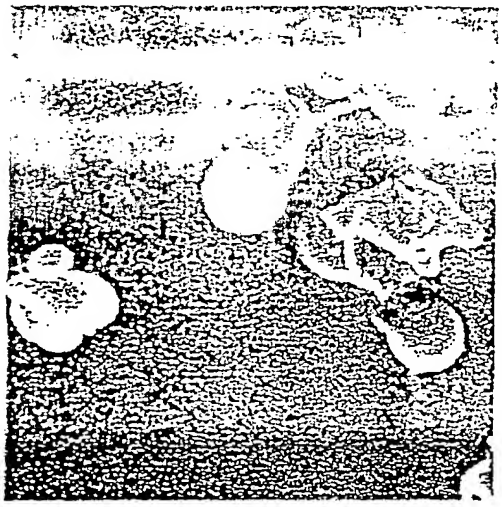
Fig. 1 Shows colonies of *Mycoplasma* species A39 (a), *fermentans* (b), *hominis* (c) and *pneumoniae* (d), grown on solid media in 95% N_2 /5% CO_2. The bar represents 1mm.

Figure 2: Morphology of MEH-A39

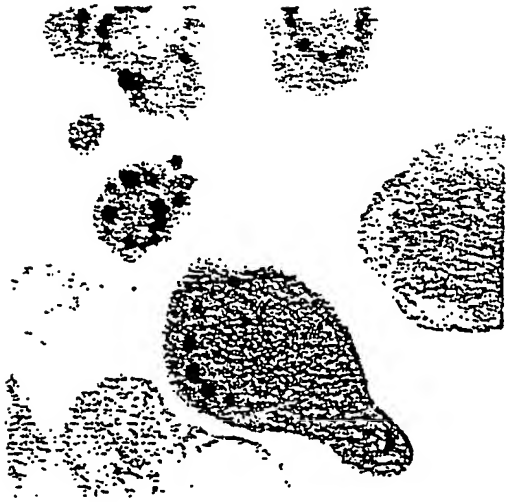
A: Colony appearance on agar



B: Cells on a 0.2µ filter



C: "Flask-shaped" cells (x 45000)



D: Cell dividing (x 35000)

Figure 3: Phylogenetic tree of 16S rDNA sequences

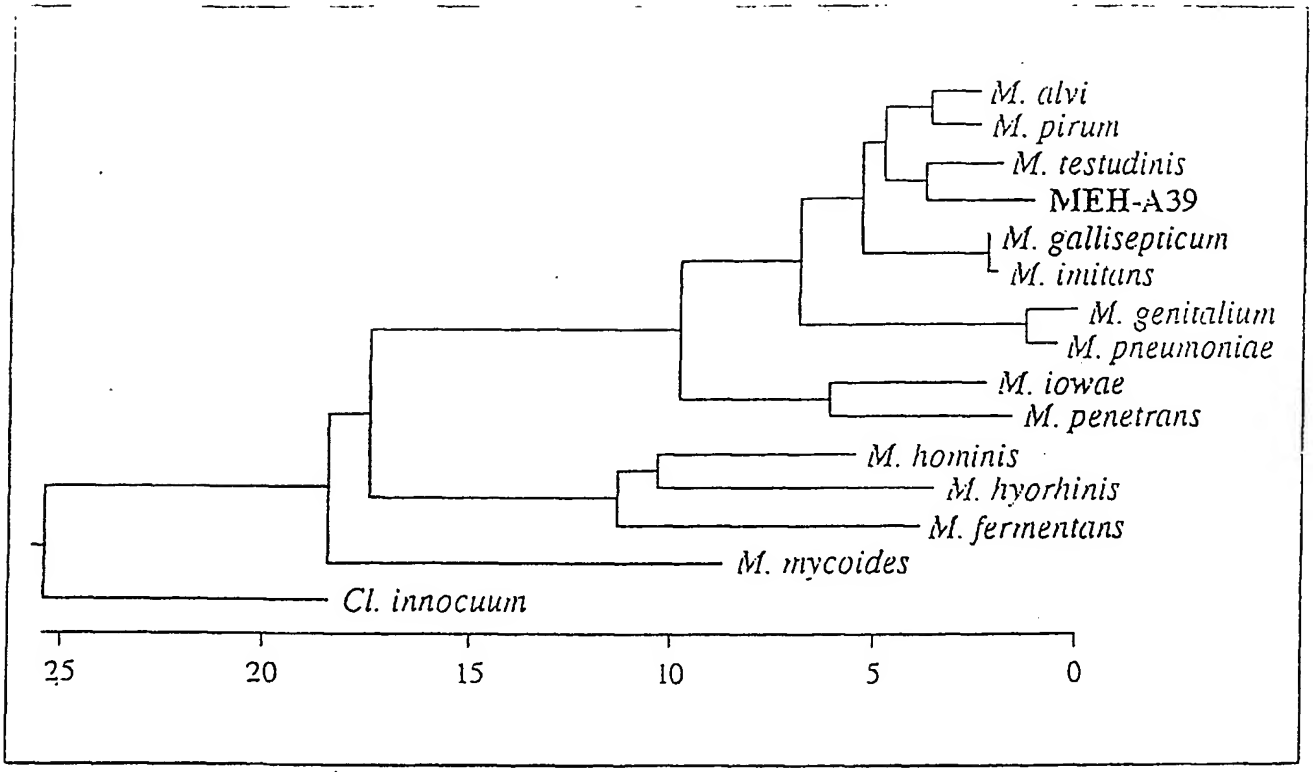
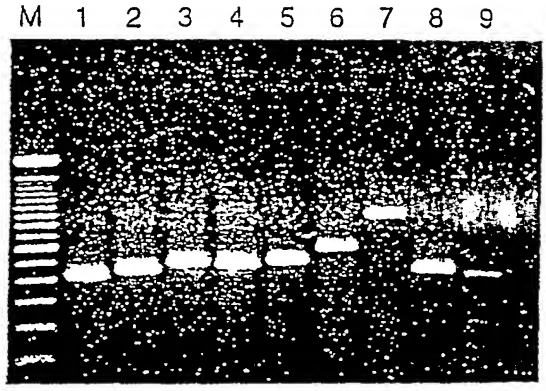


Figure 4: 16S-23S Intergenic Spacer Sequence PCR

M. 100bp ladder
1. *M. iowae* (695) NCTC 10185
2. *M. penetrans* GTU-54-6A1
3. *M. alvi* (Ilsley) NCTC 10157
4. *M. pirum* (70-159) NCTC 11702
5. *M. testudinis* (1008) NCTC 11701
6. *Mycoplasma* sp. MEH-A39
7. *M. gallisepticum* (PG31) NCTC 10115
8. *M. pneumoniae* (FH) NCTC 10119
9. *M. genitalium* (G37) NCTC 10195



A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/30 C12R1/35 C07K14/30 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C07K C12N C12R

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KONG FANRONG ET AL: "Species-specific PCR for identification of common contaminant mollicutes in cell culture." APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 67, no. 7, July 2001 (2001-07), pages 3195-3200, XP002191226 ISSN: 0099-2240	19,23, 25,26
A	page 3196, right-hand column -page 3199; figure 1; tables 1-3	13-18, 20-22,24
X	WO 99 21855 A (SANDERSON FRANCIS DOMINIC ;DABBS STEPHEN (GB); HUNT ERIC (GB); FRY) 6 May 1999 (1999-05-06) page 1, line 1 - line 19 page 15, line 5 - line 14	6-12

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2002	12/03/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	van Klompenburg, W

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 110 681 A (OVYN CAROLINE LOUISE LUCIENNE ET AL) 29 August 2000 (2000-08-29) claims 1-20; example 1	13-18

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 6-11 relate to a compound defined by reference to a desirable characteristic or property, namely being capable of treating infection by mycoplasma spp A39
The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the pleuromutilin derivatives prepared in example 1 and mentioned in the description at page 7.

Furthermore, Present claims 13-15, and claim 18 in part, relate to an oligonucleotide probe defined by reference to a desirable characteristic or property, namely being capable of hybridising to a region of polynucleotide from mycoplasma spp. A39 and incapable of hybridising to a polynucleotide from further mycoplasma.
The claims cover all oligonucleotide probes having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such oligonucleotide probes. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the oligonucleotide probe by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the oligonucleotide probes derived from 16S RNA as presented in SEQ ID NO:1 and the primers mentioned in the description at page10.

Furthermore Present claims 19,20,23-25 relate to a primer pair defined by reference to a desirable characteristic or property, namely being capable of amplifying in PCR a target nulceic acid from mycoplasma spp A39 and incapable of amplifying a sequence of the same length on the target seqeunce from further mycoplasma
The claims cover all primer pairs having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such primer pairs. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

attempt is made to define the primer pairs by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the primer pairs mentioned in the description at page 10 and those that can be derived from SEQ ID NO:1

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9921855 A	06-05-1999	AU 742167 B2	20-12-2001
		AU 9636198 A	17-05-1999
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		CN 1283197 T	07-02-2001
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		WO 9921855 A1	06-05-1999
		HU 0004040 A2	28-05-2001
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		NO 20002173 A	05-06-2000
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		TR 200001203 T2	21-08-2000
		US 6281226 B1	28-08-2001
		ZA 9809767 A	28-04-2000
US 6110681 A	29-08-2000	AU 1795297 A	16-09-1997
		WO 9732036 A1	04-09-1997
		EP 1019532 A1	19-07-2000
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